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(54) Title: USE OF SULFATED BACTERIAL POLYSACCHARIDES SUITABLE FOR THE INHIBITION OF ANGIOGENESIS

(57) Abstract: The present invention refers to the use of N,O sulfated K5 having a degree of sulfation of at least 2, and of their pharmaceutical acceptable salts for the preparation of medicaments for treating angiogenesis-dependent diseases.

"Use of sulfated bacterial polysaccharides suitable for the inhibition of angiogenesis".

Subject of the invention

It is known that angiogenesis or neovascularization can represent both a physiologic and pathologic process. Angiogenesis rarely occurs in the adults except in the female reproductive system, where it occurs in the ovary and in the endometrium during the menstrual cycle and in the placenta during pregnancy.

Moreover angiogenesis performs an important role in the inflammatory phenomena and in wound healing processes.

Said angiogenetic processes are limited in time and strictly regulated. Conversely, an angiogenetic uncontrolled process is the basis of numerous pathologies that, for this reason, are defined angiogenesis-dependent. For example in the ophthalmic field diabetic retinopathy, neovascularization of transplanted cornea, neovascular glaucoma, trachoma, retrolental fibrodysplasia are cited. In dermatology, psoriasis and pyogenic glaucoma are cited. At cardiovascular level angiogenesis is involved in the development of atherosclerotic plaque, in the hemangioma and angiofibroma and in the artero-venous malformations. Also angiogenesis was observed during arthritis.

Finally angiogenesis represents a fundamental pathogenic moment both for the development of the solid tumour and for its metastatic diffusion (Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nature Med, 1995; 1:27-31).

The growth of the new capillaries is an ordered process during which the endothelial cell produces proteolytic enzymes, moves toward the angiogenic stimulus and spreads. The new capillaries originate from the small veins or from preformed capillaries, where a local degradation of the basal membrane by proteases released by endothelial cells is observed. Because of the basal membrane dissolution the endothelial cells begin the chemotactic migration

giving rise to endothelial sprouts. The vasal sprout continuously growths because of the proliferation of the endothelial cells of the middle-distal region and the migration of the endothelial cells of the apical side. The endothelial sprouts anastomose and the endothelial cells differentiate giving rise to new vessels. (Ausprunk DH, Folkman J. Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumour angiogenesis. Microvasc. Res. 1977; 14:53-65).

All the steps of this process can be reproduced in vitro with specific experimental models and can be the target of the action of potential agents with angiostatic activity. Moreover potentially angiostatic substances can be tested in vivo in animal models such as the chick embyo chorioallantoic membrane (Auerbach W., Auerbach R. Angiogenesis inhibition: a review. Pharmac. Ther. 1994; **63**: 265-311).

Prior art

Substances such as suramine, cytocalasine D, TGF beta and genisteine are known to be useful for the inhibition of angiogenesis. These substances, however, show several drawbacks for their administration as drugs.

It is also known that the capsular polysaccharide K5 isolated from strains of *Escherichia Coli* (herein below also simply referred to as "K5") described by W.F. Vann et al. (1981) in Eur. J. Biochem 116, 359-364 shows the same sequence as the biosynthetic precursor of heparin and heparan sulfate (N-acetylheparosan) and is chemically constituted by repetitive disaccharide units formed by D-glucuronic acid and N-acetylglucosamine linked α1-4, while the disaccharide units D-glucuronyl-N-acetylglucosamine are linked β 1-4. The only difference between the heparin precursor N-acetylheparosan and K5 polysaccharide, which is not important for the biological activities of K5 and its derivatives, is the presence of a double bond in position 4(5) at the non reducing end of some chains of the polymer, as for instance described in EP

489647 and EP 544592, herein below mentioned.

After this first publication, other papers and patent applications described the preparation of the E.coli K5 polysaccharide having molecular weight ranges from few thousand to many hundred thousand Daltons. For example EP 333243, IT 1230785, EP 489647, EP 544592, WO 92/17507, WO 102597, and the paper of M. Manzoni et al. (1996), Journal Bioactive Compatible Polymers, 11, 301-311 are indicated.

Furthermore, K5 from fermentation was chemically modified to obtain heparin-like products. Thus, among the above mentioned documents, WO 92/17507, EP 489647 and EP 544592 describe N,O sulfated heparosans with low and high molecular weight having anticoagulant and antithrombotic activities, IT 1230785 and WO 92/17507 describe N-deacetylated-N,O-sulfated derivatives of K5 having a certain number of glucuronic units epimerized to iduronic units. Moreover WO 98/34958 describes O-sulfated derivatives of K5 polysaccharide with anti-metastatic and anti-viral activities. N,O sulfated derivatives of N-deacetylated K5 polysaccharide, also having anti-metastatic activity, are described in WO 98/09636. The N,O sulfated derivatives of K5 described in these references have a degree of sulfation of from 1.6 to 3.1, while the degree of sulfation of the O-sulfate K5 can reach the value of 4.

Casu et al. (1994) Carbohydrate Research, 263, 271-284 describe the N-deacetylation of K5, the N-sulfation and three methods of O-sulfation indicated as B, C and AC. According to method C, in which the sulfation of the N-sulfate K5 is performed using 10 mole equivalents of sulfating agent per free hydroxyl group at a temperature of 25-55°C for a period of time ranging from 1 to 24 hours, polysulfated compounds are obtained after a further N-sulfation having a maximum sulfate/carboxyl ratio of 3.1.

Herein below the K5 derivatives are also designated as follows: "N-deacetylated K5" the N-deacetylated K5 polysaccharide, "N-sulfate K5" the

N-deacetylated-N-sulfated K5 polysaccharide, "N,O-sulfate K5" the N-deacetylated-N,O-sulfated K5 polysaccharide and "N,O-oversulfated K5" the N-deacetylated-N,O-sulfated K5 polysaccharide with high degree of sulfation, obtainable according to the Method C of the above mentioned paper Casu et al. (1994).

The expression "O-oversulfation conditions" indicates condition of exhaustive O-sulfation, like that of the above mentioned Method C, with from 2 to 10 moles of free SO_3/OH at 25-55°C for 1- 24 hours. By "degree of sulfation " the number of sulfate groups per disaccharide unit, expressed as sulfate/carboxyl ratio (SO_3^-/COO^-) determined according to Casu et al., 1975, Carbohydrate Research, 39, 168-176 is designated.

The invention is based on the hypothesis that N,O sulfate polysaccharides from K5 can have high anti-angiogenetic activity, with low hemorrhagic risk and that said N,O sulfated K5 can be used in the treatment of the angiogenesis-dependent pathologies.

Summary of the invention

It was found that N,O sulfated K5 having degree of sulfation of at least 2 exert a marked in vivo antiangiogenetic activity with a favorable ratio with respect to the global anticoagulant activity.

It was also found that, by purifying K5, obtained by fermentation, by treatment with isopropanol in a highly saline solution, a pure K5 polysaccharide substantially free of lipophilic substances and, submitting said K5 free of lipophilic substances to N-deacetylation, N-sulfation, O-sulfation in the conditions of oversulfation and, eventually, to a new N-sulfation, N,O oversulfated K5 are obtained with a degree of sulfation higher than 3.2. Also these N,O oversulfated K5 have high in vivo antiangiogenetic activity with favorable ratio with respect to the global anticoagulant activity.

Said N,O sulfate K5 having degree of sulfation of at least 2 can then be used in the preparation of pharmaceutical compositions suitable for the treatment of

angiogenesis-dependent pathologies at doses in which the hemorrhagic side effect is extremely reduced.

Detailed description of the invention

Thus, according to one of its aspects, the present invention refers to the use of N,O sulfated K5 having a degree of sulfation of at least 2, and of their pharmaceutical acceptable salts for the preparation of medicaments for combating the angiogenesis-dependent pathologies.

In particular, the N,O sulfated K5 with a degree of sulfation of at least 2 and their salts have a high affinity for the growth factors such as the fibroblast growth factor (FGF2) and exert a marked antiangiogenetic activity in vitro and in vivo. These compounds are active in the inhibition of all the steps of angiogenesis and, in particular, they inhibit the binding of the angiogenetic growth factor FGF2 to its high affinity receptors and to the heparan sulfate proteoglycans of the cellular surface, the FGF2 induced proliferation on endothelial cells in culture, the formation of pluricellular sprouts in fibrin gel and the differentiation on extracellular matrix of endothelial cells. Moreover, they inhibit the neovascularization on the chick embryo chorioallanthoic membrane in vivo.

Essential condition for a product to be considered a good antiangiogenetic drug is that it shows a good activity in all the tests. The N,O sulfated K5 with a degree of sulfation of at least 2, or anyway of from 2 to 4, especially those with degree of sulfation of from 2.5 to 4, in particular the N,O oversulfated K5 with degree of sulfation of from 3.2 to 4, advantageously from 3.5 to 4, preferably from 3.7 to 4, are generally active in all the above mentioned tests and are herein below globally indicated as "active ingredients" of the invention. The expression "active ingredients" includes also the pharmaceutical acceptable salts of the above illustrated N,O sulfated K5 with a degree of sulfation of at least 2.

The different steps of angiogenesis are inhibited by the active ingredients of

the present invention at concentrations of from 0.01 to 100 μg/ml in the in vitro tests and at a dose comprised between 10 and 50 μg per implant on choroallantioic membrane.

Thus, according to another of its aspects, the present invention concerns a method to treat the angiogenesis-dependent pathologies in mammals which comprises administering, to a mammal in need of said treatment, an effective amount of a N,O sulfated K5 having a degree of sulfation of at least 2, or of a pharmaceutically acceptable salt thereof. More particularly, the N,O sulfated K5 used for this method, will have a degree of sulfation of from 2.5 to 4, or will be a N,O oversulfated K5 with a degree of sulfation of from 3.2 to 4, advantageously from 3.5 to 4, preferably from 3.7 to 4.

Among the salts of the above mentioned N,O sulfated K5, the sodium, potassium, calcium, magnesium, aluminum and zinc salts are the preferred.

The active ingredients of the invention preferably show mean molecular weights comprises between about 2,000 and about 65,000, advantageously between 2,500 and 20,000 dalton (D). All the molecular weights indicated are intended to be expressed in dalton and calculated according to Harenberg et al. Journal Chromatography (1983), 261, 287-292.

Angiogenesis-dependent pathologies that can be treated with the active ingredients of the invention are for example, among those found in the human beings, diabetic retinopathy, neovascularization of the transplanted cornea, neovascular glaucoma, trachoma, retrolental fibrodisplasia, psoriasis, pyogenic glaucoma, development of the ateroschlerotic plaque, hemangioma and angiofibroma, artero-venous malformations, arthritis, and, in the combinatorial therapy, solid tumors.

The N,O oversulfated K5 having a degree of sulfation of from 3.2 to 4 are prepared by a process which comprises

(a) treating a K5 from fermentation with isopropanol in a highly saline

solution;

(b) submitting the thus purified K5 to a N-deacetylation by alkaline hydrolysis and to a subsequent N-sulfation by treatment with a N-sulfating agent;

(c) treating an ammonium salt of the N-sulfate K5 thus obtained with an O-sulfating agent in the O-oversulfation conditions;

(d) if needed, submitting the product thus obtained to a N-sulfation and isolating the N,O oversulfated K5 as sodium salt which, optionally, is converted into another salt.

In step (a), the K5 starting material can be one of the products obtained by fermentation of wild or cloned *Escherichia coli* strains, producing K5. In particular the K5 described in literature like those above cited can be used, advantageously those described by M.Manzoni et al., Journal Bioactive and Compatible polymers, 1996, 11, 301-311 and the one illustrated in PREPARATION I herein below.

More advantageously, the K5 starting material has a low molecular weight, in particular with a molecular weight distribution of from about 1,500 to about 15,000, preferably from about 2,000 to about 9,000, with a mean molecular weight of about 5,000, or a higher mean molecular weight, in particular with a distribution of from about 10,000 to about 50,000, preferably from about 20,000 to about 40,000, with a mean molecular weight of about 30,000. Preferably, the K5 starting material has a distribution of molecular weight of from about 1,500 to about 50,000 with a mean molecular weight of 20,000-25,000.

The molecular weight of K5 and of the herein described derivatives thereof is intended as calculated using heparin fractions of known molecular weight as standards.

The starting product can be a previously purified K5 from which, for example, the endotoxins, the pyrogens or other impurities have been eliminated by

known methodologies.

Likely, if the K5 obtained at the end of step (a) is used for pharmaceutical purposes or for the preparation of N,O sulfated K5 for pharmaceutical use, it can be purified from pyrogens and endotoxins.

Practically, the starting K5 is dissolved in a 2-5 M saline solution, preferably of sodium chloride, at a concentration of from 0.5 to 10% and treated with 1-3 volumes of isopropanol at a temperature of 0-8°C and the thus obtained solution, brought to 2-4 M by a further addition of salt, preferably sodium chloride.

After 1-18 hours at the same temperature, the product of step (a) completely precipitates and is isolated by filtration or centrifugation. If the purity of the product is not satisfactory, the procedure of step (a) is repeated. The solid product thus obtained is redissolved in water and recovered by ultrafiltration on a membrane.

At the end of step (a) a K5 having the same characteristics as those of the starting material, but being substantially free of lipophilic substances is obtained.

Practically, the K5 free of lipophilic substances is obtainable by a process which comprises (a1) treating a K5 from fermentation, dissolved in a 4 M solution of sodium chloride at 4°C with 1 volume of isopropanol, (a2) bringing the saline solution to 3 M by adding the calculated amount of a sodium chloride saturated solution, (a3) letting the solution to stay at 4°C overnight and (a4) isolating the product by centrifugation and eliminating the salts by ultrafiltration.

By the purification with isopropanol it is thus possible to obtain a K5 free of lipophilic substances with a purity higher than 99%. This K5 allows to obtain a high O-sulfation in the next step (c).

In step (b), the N-deacetylation is performed according to the known methods of alkaline hydrolysis, for example with hydrazine sulfate in hydrazine or with

a base such as an alkaline hydroxide, for example sodium or potassium hydroxide, in water. Preferably the reaction is performed in an aqueous solution of sodium hydroxide at a temperature of 40-80°C, by controlling the course of the reaction. In general, after at most 30 hours, but practically after 12-24 hours the N-deacetylation is complete and the alkalinity of the medium is neutralized by treatment with an acid, preferably hydrochloric acid.

The solution containing the K5 and the salts is subsequently treated with a N-sulfating agent such as the adduct of a tertiary organic base with a sulfuric anhydride (sulfur trioxide), such as pyridine sulfur trioxide ($C_5H_5N.SO_3$) or a trialkylamine sulfur trioxide such as trimethylamine sulfur trioxide in the presence of an alkaline carbonate such as sodium carbonate. The reaction can be performed at room temperature (20-30°C), but it is also possible to work at higher temperatures (till 65°C) to shorten the reaction time. The addition of the alkaline carbonate and of the sulfating agent can be performed concurrently or the alkaline carbonate is introduced in bulk and the sulfating agent subsequently, stepwise, in a period of time which can last from 5 minutes to 12 hours. At the end of the reaction the mixture, at room temperature, is brought to pH 7.5-8 with an acid, preferably hydrochloric acid and the salts are eliminated for example by diafiltration. The so obtained solution, containing the N-sulfate K5 as an alkaline salt, can be passed to the subsequent step (c), or it can be concentrated and the N-sulfate K5 can be isolated as sodium salt with conventional methods. The thus obtained N-sulfate K5 is 90-100% sulfated.

In step (c) a solution containing the alkaline N-sulfate K5 obtained in step (b) is neutralized for example by passage on a cationic exchange resin, like IR 120 H^+ till acid pH. The acidic solution so obtained is treated with a tertiary or quaternary organic base, for example with a trialkylamine like tributylamine, or with the hydroxide of a tetralkylammonium, preferably tetrabutylammonium hydroxide, reduced to the minimum volume and freeze

dried. The thus isolated ammonium salt of the N-sulfate K5 is suspended in a polar aprotic solvent such as dimethylformamide or dimethylsulfoxide and treated with an O-sulfating agent, for example with the adduct $C_5H_5N.SO_3$. The adduct $C_5H_5N.SO_3$ can be used either in the solid state or in solution in the same polar aprotic solvent. The sulfation is performed at a temperature that can vary from the room temperature (20-30°C) to 70°C, preferably from 40 to 60°C, for a period of time of from 2 to 24 hours.

At the end of the reaction, the solution at room temperature is treated with sodium chloride saturated acetone till complete precipitation. The precipitate is separated from the solvent by filtration, dissolved in the minimum amount of deionized water, for example 100 ml, and sodium chloride is added to the solution till 0.2 M concentration. The solution is brought to pH 7.5-8 with 2N sodium hydroxide and treated with acetone till complete precipitation. After filtration the solid is dissolved in 100 ml of deionized water and purified from the residual salts by ultrafiltration as described in step (b).

If from the analysis by ^{13}C-NMR of a freeze dried sample of the thus obtained product a partial N-desulfation occurred during the oversulfation, the product is submitted to step (d).

In step (d) the product obtained at the end of step (c) is treated with a N-sulfating agent by operating under the conditions of step (b) till complete N-sulfation, repeating the procedure if the N-sulfation is not complete.

The N,O oversulfated K5 thus obtained is isolated as sodium salt, that can be converted into another salt, like potassium, calcium, magnesium, aluminum, zinc or complex salts using known methods, for example by ionic exchange with a suitable resin, by precipitation with solvents or by ultrafiltration with membranes.

The purity of the new purified K5 from fermentation can be assayed by ^1H-NMR spectrum, by UV spectrum, by carbazole reaction, or by a kit for the protein determination. By these assays it was demonstrated that the K5

obtained at the end of step (a) has, as essential characteristic, a ^{1}H-NMR spectrum in which the signals in the field below 1.5 ppm are absent. Moreover the nucleic acids are not detectable, (absorbance 0 at 260 nm with a standard UV spectrophotometer) and the proteins are not higher than 0.5%, advantageously below 0.25%, more advantageously below 0.1%, preferably below 0.03% according to BioRad kit.

Actually, the new pure K5 obtained at the end of step (a) is free from lipophilic substances and nucleic acids. The use of "substantially", referred to the absence of lipophilic substances and of "not detectable" referred to the nucleic acids takes in account the sensitivity of the instruments used which have not revealed the presence of the above mentioned impurities.

Thus it was established that the ^{1}H-NMR spectrum of the pure K5 polysaccharide obtained in this way lacks the signals at <1.5 ppm characteristics of the methyl group of lipophilic substances.

The new thus purified K5 compounds, which allow the preparation of N,O oversulfated K5 with a high degree of sulfation, have preferably a low molecular weight, in particular with a distribution of from about 1,500 to about 15,000, preferably from about 2,000 to about 9,000, with a mean molecular weight of about 5,000, or a higher molecular weight, in particular with a distribution of from about 10,000 to about 50,000, preferably from about 20,000 to about 40,000 with a mean molecular weight of about 30,000. Preferably the K5 starting material has a molecular weight distribution of from about 1,500 to about 50,000 with a mean molecular weight of 20,000-25,000.

The N,O sulfated K5 with degree of sulfation higher than 2, as above illustrated, especially as their pharmaceutically acceptable salt form, are active ingredients of pharmaceutical compositions useful for the treatment of angiogenesis-dependent pathologies.

Advantageously said N,O sulfated K5 have a low molecular weight, in particular with a distribution of from about 2,000 to about 16,000, preferably

from about 2,500 to about 10,000 with a mean molecular weight of about 6,500, or a somewhat higher molecular weight, in particular with a distribution of from about 13,000 to about 65,000, preferably from about 25,000 to about 50,000 with a mean molecular weight of about 40,000. Preferably the N,O oversulfated K5 starting material of the present invention has a molecular weight distribution of from about 2,000 to about 65,000, with a mean molecular weight of 25,000-30,000. Also N,O oversulfated K5 having a very low mean molecular weight, for example of from about 2,000 to 5,000, obtained by depolymerization, constitute very interesting products.

In the case of N,O oversulfated K5 the depolymerization that allows the preparation of the products with mean molecular weight of from 2,000 to 5,000 can be performed at the end of one of steps (b)-(d) of the process illustrated above, preferably at the end of step (b) or on the final N,O oversulfated K5.

The depolymerization can be performed according to anyone of the known methods for the depolymerization of heparin, for example by nitrous acid and subsequent reduction with sodium borohydride (EP 37319), by periodate (EP 287477), by free radicals (EP 121067) or by β-elimination (EP 40144). In the case of N,O oversulfated K5 the depolymerization is advantageously performed on a N-sulfate K5 obtained at the end of step (b) with nitrous acid and subsequent reduction with sodium borohydride as detailed in EP 544592. At the end of the depolymerization and reduction, the low molecular weight product thus obtained is submitted to steps (c) and, optionally, (d) and the N,O oversulfated K5 is isolated.

Among the salts of the above illustrated N,O sulfated K5 with degree of sulfation of at least 2, the sodium, potassium, calcium, magnesium aluminum and zinc salts are preferred active ingredients.

For the foreseen therapeutic uses, the active ingredients of the present

invention and their salts are formulated according to conventional techniques in suitable administration forms such as for example sterile solutions, topic dosage forms, and, in general, in all those forms proposed till today for polysaccharide or glycosaminoglycan derivatives. Also the therapeutic doses are chosen in analogy to those already studied for the known natural compounds.

The administration of the active ingredient can be performed by oral, transdermic or preferably parenteral route, in particular subcutaneous, intramuscular or intravenous or topic or transmucosal route, such as for example nasal administration.

In humans, the daily dose for the parenteral administration is provided as 0.5-500 mg /Kg/die, advantageously in 5-250 mg/Kg/die, preferably in 10-150 mg/Kg/die, while the dose provided by topic route is 1-1,000 mg/Kg/die, advantageously 10-500 mg/Kg/die, preferably 20-100 mg/Kg/die.

For their administration, the active ingredients of the invention are formulated in pharmaceutical compositions suitable for the treatment of the angiogenesis-dependent pathologies like those above mentioned.

Thus, according to another of its aspects, the present invention provides pharmaceutical composition for the treatment of angiogenesis-dependent pathologies which comprises, as one of its active ingredient, a pharmacologically effective amount of a N,O sulfated K5 having a degree of sulfation of at least 2, or of one of its pharmaceutically acceptable salts, preferably in admixture with a carrier or a pharmaceutical excipient.

Said N,O sulfated K5 can have one of the other above illustrated characteristics of degree of sulfation or of molecular weight.

A salt chosen in the group comprising the sodium, potassium, calcium, magnesium, aluminum and zinc salts constitutes valid active ingredient of the compositions of the present invention.

In the pharmaceutical compositions of the present invention for the oral,

subcutaneous, intravenous, intramuscular, transdermic, transmucosal, or topical administration, the active ingredients are preferably administered as dosage unit, in admixture with the classical pharmaceutical excipients or carriers. The dose can amply change in function of age, weight, and health conditions of the patient, as much as of severity of the infection and of route of administration. This dose comprises the administration of a dosage unit from 1 to 1,000 mg, advantageously from 10 to 750 mg, preferably from 250 to 500 mg, from one to three times per day by intravenous, intramuscular, subcutaneous, oral, transdermic, transmucosal or topic route.

According to another of its aspects, the invention provides a method for the treatment of the angiogenesis-dependent pathologies, which comprises the administration to the patient in need of such treatment an effective amount of a N,O sulfated K5 having a degree of sulfation of at least 2, especially from 2.5 to 4, advantageously an effective amount of a N,O oversulfated K5 having a degree of sulfation from 3.2 to 4, advantageously from 3.5 to 4, preferably from 3.7 to 4 or of one of its pharmaceutically acceptable salts.

The method of the invention is carried out by administering a pharmaceutical composition containing an active ingredient according to the invention in dosage unit and at the daily doses such as those illustrated above.

Advantageously the method of the invention provides a dose of from 0.5 to 500 mg/Kg/die for parenteral administration and a dose of from 1 to 1,000 mg/Kg/die for topical administration.

The following examples illustrate the invention.

PREPARATION I

Preparation of the K5 polysaccharide from Escherichia Coli

First a fermentation in flask using the following medium is performed:

Defatted soy	2 g/l
K_2HPO_4	9.7 g/l
KH_2PO_4	2 g/l

$MgCl_2$	0.11 g/l
Sodium citrate	0.5 g/l
Ammonium sulfate	1 g/l
Glucose	2 g/l
Water	1,000 ml
pH = 7.3	

The medium is sterilized at 120 °C for 20 minutes. The glucose is prepared separately as a solution which is sterilized at 120°C for 30 minutes and sterile added to the medium. The flask is inoculated with a suspension of E.Coli cells Bi 8337/41 (O10:K5:H4) from a slant maintained in Tryptic soy agar, and incubated at 37°C for 24 hours under controlled stirring (160 rpm, 6 cm of run). The bacterial growth is measured counting the cells with a microscope. In a further step, a Chemap-Braun fermentor with a volume of 14 litres containing the same medium above is inoculated with the 0.1% of the above flask culture and the fermentation is performed with 1vvm aeration (vvm = air volume for liquid volume for minute), 400 rpm stirring and temperature of 37°C for 18 hours. During the fermentation pH, oxygen, residual glucose, produced K5 polysaccharide and bacterial growth are measured. At the end of the fermentation the temperature is raised to 80°C for 10 minutes. The cells are separated from the medium by centrifugation at 10,000 rpm and the supernatant is ultrafiltrated through a SS316 (MST) module equipped with PES membranes with a nominal cut off of 800 and 10,000 D to reduce the volume to 1/5. Then K5 polysaccharide is precipitated adding 4 volumes of acetone at 4°C and left to sediment for one night at 4°C and finally is centrifuged at 10,000 rpm for 20 minutes or filtrated. Then a deproteinization using a protease of the type II from *Aspergillus Orizae* in 0.1M NaCl and 0.15 M ethylenediaminotetracetic acid (EDTA) at pH 8 containing 0.5% sodium dodecyl sulfate (SDS) (10 mg/l of filtrate) at 37°C for 90 minutes is performed. The solution is ultrafiltrated on a SS 316 module with a nominal

cut off membrane of 10,000 D with 2 extractions with 1M NaCl and washed with water until the absorbance disappears in the ultrafiltrate. K5 polysaccharide is then precipitated with acetone and a yield of 850 mg / l of fermentor is obtained. The purity of the polysaccharide is measured by uronic acid determination (carbazole method), proton and carbon NMR, UV and protein content. The purity is higher than 80%. The so obtained polysaccharide is composed of two fractions with different molecular weight, 30,000 and 5,000 D respectively as obtained from the HPLC determination using a 75 HR Pharmacia column and one single fraction with retention time of about 9 minutes using two columns of Bio-sil SEC 250 in series (BioRad) and Na_2SO_4 as mobile phase at room temperature and flow rate of 0.5 ml/minute. The determination is performed against a curve obtained with heparin fractions with known molecular weight.

The ^{1}H-NMR spectrum of thus obtained K5 shows that in the region below 1.5 ppm a many signals attributable to the methyls of lipophilic substances are present.

PREPARATION II

Preparation of a K5 free from lipophilic substances

In 100 ml of an aqueous solution containing 4M sodium chloride and thermostated at 4°C are dissolved 5 g of the K5 obtained at the end of PREPARATION I and 1 volume of cold isopropanol is added to the thus obtained solution. The salt concentration of the solution is brought to 3 M adding a calculated amount of a saturated solution of sodium chloride and the cooled solution is kept at cold temperature (about 4°C) overnight. The precipitate formed is separated by centrifugation at 10,000 rpm for 20 minutes and the purity of the product is controlled by dialysis for one night and subsequent ^{1}H-NMR analysis from which the signals in the region below 1.5 ppm are absent. If necessary, the procedure of dissolution in water containing 4M NaCl and precipitation with isopropanol is repeated. The precipitate is

dissolved in water and ultrafiltrated on a Miniplate membrane Millipore with a 10,000 D cut off till disappearance of the salts. A K5 having a purity of at least 99% and whose ^{1}H-NMR spectrum there are no traces of lipophilic impurities in the region below 1.5 ppm.

The protein content calculated using BioRad kit is 0.02% and the nucleic acids are not detectable (absorbance 0 at 260 nm).

PREPARATION III

Preparation of a N,O oversulfated K5

(i) N-deacetylation

Ten grams of pure K5 polysaccharide prepared as described in PREPARATION I and purified as described in PREPARATION II are dissolved with 1,000 ml of 2 N sodium hydroxide and the solution thus prepared is kept at 60°C for 24 hours. The solution is brought to room temperature and then to neutral pH with 6N hydrochloric acid.

(ii) N-sulfation

To the solution containing the deacetylated K5, kept at 40°C, 16 g of sodium carbonate and subsequently 16 g of pyridine sulfur trioxide in 4 hours are added. At the end of the reaction, after 24 hours, the solution is brought to room temperature and then to pH 7.5-8 with a 5% solution of hydrochloric acid. The product is purified from salts by diafiltration using a spiral membrane of 1,000 D (Prepscale Cartridge-Millipore). The process is ended when the conductivity of the permeate is below 1,000 μS, preferably below 100 μS. The intradialysis is reduced till a polysaccharide concentration of 10% using the same dialysis system in concentration. The concentrated solution is freeze dried. The analysis of the ^{13}C-NMR does not show N-acetyl or NH_2 residual groups.

(iii) O-oversulfation

The freeze dried product obtained at the end of step (ii) is dissolved in 100 ml

of deionized water and the solution is brought to 10°C with a cooling bath then passed onto a cationic exchange resin IR120H$^+$ (100 ml). Both the column and the reservoir are kept at 10°C. After the passage of the solution containing the sample the resin is washed with deionized water till the pH of the permeate is higher than 6 (about 3 volumes of deionized water). The acid solution is brought to neutrality (pH 7) with tetrabutylammonium hydroxide (15% aqueous solution), then reduced to the minimum volume and freeze dried. The tetrabutylammonium salt is dissolved in 400 ml of dimethylformamide and added with 35 g of $C_5H_5N.SO_3$ in solid form. The solution is kept at 50°C for 24 hours. At the end of the reaction the solution is cooled to room temperature and added with 3 volumes of sodium chloride saturated acetone, cooled to 4°C till complete precipitation (12 hours). The precipitate is separated from the solvent by filtration, solubilized with the minimum amount of deionized water (about 100 ml) and to the solution sodium chloride till 0.2 M concentration is added.

The solution is brought to pH 7.5-8 with 2N sodium hydroxide and treated with 2 volumes of acetone till complete precipitation. The precipitate is separated from the solvent by filtration. The solid obtained is solubilized with 100 ml of deionized water and purified from the residual salts by ultrafiltration as described in step (ii) using a spiral membrane of 1,000 D (Prepscale Cartridge Millipore).

(iv) N-sulfation

The solution thus obtained, containing the O-sulfated product, is treated as previously described in step (ii) for the N- sulfation. The product shows a mean molecular weight of 15,000 D and a sulfate/carboxyl ratio of 3.84. The distribution of the sulfate groups, determined with the ^{13}C-NMR is the following: the glucosamine unit of the constitutive disaccharide is 100% N-sulfated and 6-O sulfated, while, for what concerns the glucuronic units, 30% are monosulfated and 70% are disulfated.

PREPARATION IV

Preparation of a N,O oversulfated K5

By operating as described in PREPARATION III starting from a K5 obtained and characterized as described by M.Manzoni et al. (1996), purified as described in PREPARATION II, a N,O oversulfated K5, designated K5-N,OS(H1), having mean molecular weight of 13,000 and a sulfate to carboxyl ratio of 3.54 is obtained.

PREPARATION V

Preparation of a N,O sulfated K5

By operating as described in PREPARATION III, but using 7 g of adduct $C_5H_5N.SO_3$ in step (iii), a N,O sulfated K5 designated K5-N,OS(L) is obtained, having a mean molecular weight of 14,000 and a sulfate to carboxyl ratio of 1.7.

PREPARATION VI

Preparation of a O-oversulfated K5

By operating as described in step (iii) of PREPARATION III on a K5 obtained as described in PREPARATION I and purified as described in PREPARATION II, an O-oversulfated K5, designated as K5-OS(H), having a mean molecular weight of 18,000 and a sulfate/carboxyl ratio of 3.77 is obtained.

Example 1

An active ingredient representative of the invention, the N,O oversulfated K5 obtained according to PREPARATION III, having a degree of sulfation of 3.84 and designated as K5-N,OS(H), was compared with two other derivatives of K5 polysaccharide, in particular with a N,O sulfated K5, having a degree of sulfation of 1.7 and designated as K5-N,OS(L), obtained as described in PREPARATION V, and with a O-oversulfated K5, having a degree of sulfation of 3.77, designated K5-OS(H) and prepared as described in PREPARATION VI. The products under examination were submitted to 6

different tests.

Test 1. Inhibition of the formation of the ternary complex HSPG/FGF2/FGFR1

The inhibition of the formation of the ternary complex HSPG/FGF2/FGFR1 was studied with an intercellular adhesion assay as described by LIEKENS S., LEALI D., NEYTS J., ESNOUF R., RUSNATI M., DELL'ERA P., MAUDGAL PC., DE CLERCQ E., and PRESTA M. Modulation of fibroblast growth factor-2 receptor binding, signaling, and mitogenic activity by heparin-mimicking polysulfonated compounds. Mol. Pharmacol. (1999), 56:204-213. Briefly, CHO-K1 cells are seeded in 24-well plates at the density of 90,000 cells/cm^2. After 24 hours the cells are fixed in 3% glutaraldehyde in PBS for 2 hours at 4°C and washed with 0.1 M glycine/PBS. 745 CHO *flg*-1A cells transfected with FGRF-1 are then seeded on the fixed monolayers at the density of 50,000 cells /cm^2 in DMEM containing 10 mM EDTA, 30 ng/ml FGF2, and increasing concentrations of the compounds K5-N,OS(H), K5N,OS(L), and K5-OS(H). After 2 hours of incubation at 37°C, the monosubstrate unattached cells are removed by washing with PBS and the bound cells are counted under an inverted microscope. The data are expressed as percent of the number of attached cells compared to those counted in absence of the compound under examination.

The compounds K5-N,OS(H) and K5-N,OS(L) inhibit the intercellular adhesion mediated by the formation of the ternary complex HSPG/FGF2/FGFR1 more efficiently than the compound K5-OS(H).

The results of this test are reported in Table 1, column 2.

Test 2. Inhibition of the binding of FGF2 to the endothelial cells.

Bovine aortic endothelial cells are seeded in 24-wells plates at the density of 80,000 cells/cm^2 in MEM Eagle medium containing 10% bovine fetal serum (FCS) and are then incubated for 24 hours at 37°C. At the end of the incubation the cells attached to the wells are washed twice with MEM Eagle

without FCS and then incubated for 2 hours at 4°C in MEM-Eagle containing 0.15% gelatin, 20 mM HEPES pH 7.5, 30 ng/ml ^{125}I-FGF-2 in absence and in presence of 10 μg/ml of the derivatives under examination. At the end of the incubation the radioactivity associated to the high (FGFR) and low (HSPG) affinity receptors is evaluated as previously described (MOSCATELLI, D (1992) J Biol Chem **267**:25803-25809). Briefly, the radioactivity associated to HSPGs is removed by washing twice the cellular monolayer with 20 mM HEPES pH 7.5 containing 2M NaCl, while the radioactivity associated to the FGFRs is subsequently recovered by washing twice with 20 mM sodium acetate buffer pH 4.5 containing 2M NaCl. The aspecific binding is determined by incubating the cells with ^{125}I-FGF-2 as described in presence of an excess of 1mg/ml of cold FGF-2.

The three derivatives studied inhibit the binding of ^{125}I-FGF-2 to its receptors FGFR and HSPG with the same efficacy.

The results are reported in Table 1, column 3.

Test 3. Inhibition of cell proliferation

Human endothelial cells from umbilical cord (HUVEC) are seeded in 96 well plates at the concentration of 2,500 cells/well in complete EGM medium and are incubated for 24 hours at 37°C. At the end of the incubation, the cells attached to the wells are washed twice with the medium and then incubated for further 72 hours at 37°C in EGM-2 containing 2% fetal calf serum (FCS) in absence and in presence of FGF-2 (30 ng/ml) and of increasing concentration of the three compounds. At the end of the incubation the cells are stained with crystal-violet and the plates are read with an ELISA plate reader at the wavelength of 595 nm.

Only the K5-N,OS(H) derivative significantly inhibits cell proliferation.

The results are shown in Table 1, column 4.

Test 4. Inhibition of the formation of endothelial sprouts in fibrin gel

The inhibition of the formation of endothelial sprouts in fibrin gel is evaluated as described by GUALANDRIS A, M. RUSNATI, M. BELLERI, E.E. NELLI, M. BASTAKI, M.P. MOLINARI-TOSATTI, F. BONARDI, S. PAROLINI, A. ALBINI, L. MORBIDELLI, M. ZICHE, A. CORALLINI, L. POSSATI, A. VACCA, D. RIBATTI and M. PRESTA, Cell Growth & Diff. (1996), 7, 147-160. Briefly, MAE-3F2T cells are seeded on agarose gel at the density of 75,000 cells/cm^2 in a medium containing 10% FCS to induce the formation of cell aggregates (spheroids). After 16 hours the spheroids are collected and incorporated in a 2.5% fibrin gel containing aprotinin (50 U/ml). The mixture is then seeded in 48 well plates (0.25 ml/well) and left to gel by adding thrombin for 10 minutes at 37°C. At the end the three derivatives are added at the concentration of 100 μg/ml in a medium containing 10% FCS and aprotinin. The cells are observed after 24 hours from the beginning of the treatment.

The derivatives K5-OS(H) and K5-N,OS(H) completely inhibit the formation of the endothelial sprouts, while K5-N,OS(L) is ineffective.

The results are shown in Table 1, column 5.

Test 5. Inhibition of the endothelial differentiation on Matrigel

The inhibition of the endothelial differentiation on Matrigel is evaluated as described by GUALANDRIS A, M. RUSNATI, M. BELLERI, E.E. NELLI, M. BASTAKI, M.P. MOLINARI-TOSATTI, F. BONARDI, S. PAROLINI, A. ALBINI, L. MORBIDELLI, M. ZICHE, A. CORALLINI, L. POSSATI, A. VACCA, D. RIBATTI and M. PRESTA, Cell Growth & Diff. (1996), 7, 147-160. Briefly, MAE-3F2T cells are seeded on Matrigel in 48 well plates at the density of 75,000 cells/cm^2 in a medium with 10% FCS. After 20 minutes from the seeding the derivatives under examination are added to the medium at the concentration of 100 μg/ml. The cells are observed at the microscope during the further 24 hours.

The products K5-OS(H) and K5-N,OS(H) completely inhibit the endothelial differentiation, while K5-N,OS(L) is ineffective.

The results are shown in Table 1, column 6.

Tets 6. Inhibition of angiogenesis in vivo on chick embryo choroallantioic membrane (CAM)

Sponge implants of Gelfoam (Upjohn) are applied on chick embryo CAM at the 8th day of development according to RIBATTI D, GUALANDRIS A, BASTAKI M, IURLARO M, RONCALI L and PRESTA M., J. Vasc. Res. (1997), 34, 455-463. Immediately after the application 5 µl of a solution of saline containing 50 µg of the K5 derivative are pipetted. The sponges are examined daily till the 12th day of incubation. The quantification of the angiogenesis is obtained by counting of the number of the macroscopic vessels observed around the sponge at the different days of development.

The derivative K5-N,OS(H) significantly inhibits angiogenesis of CAM, while the derivatives K5-OS(H) and K5-N,OS(L) are ineffective.

The results are shown in Table 1, column 7.

Table 1 – Biological activity of the analyzed products:

compound	Test 1 ID50	Test 2 FGFR HSPG	Test 3 ID50	Test 4	Test 5	Test 6
K5-N,OS(H)	1.0 ng/ml	65% 78%	30 µg/ml	100 %	100%	70%
K5-N,OS(L)	3.0 ng/ml	78% 95%	>>300 µg/ml	0%	0%	0%
K5-OS(H)	100.0 ng/ml	63% 80%	>>300 µg/ml	100 %	100%	0%

The results reported in said table show that the compound K5-N,OS(H), representative of the present invention, is the sole product which is active in all of the tests and is the most active one in test 1,3 and 6.

Example 2

Determination of the global anticoagulant activity

The global anticoagulant activity was evaluated in plasma for the N,O sulfated K5 representative of the present invention, i.e. K5-N,OS(H) tested in example 1, by aPTT according to Andersson et al. (1976), Thrombosis Res., 9, 575 against the IV International Standard of heparin. The sample has an activity of 40 IU (International Units)/mg compared to 200 IU/mg of standard heparin.

Claims

1. Use of a N,O-sulfated K5 having a degree of sulfation of at least 2 or of a pharmaceutically acceptable salt thereof for the preparation of pharmaceutical compositions for combating angiogenesis-dependent pathologies.

2. Use according to claim 1 wherein said N,O-sulfated K5 has a degree of sulfation of from 2.5 to 4.

3. Use according to claim 1 wherein said N,O-sulfated K5 is a N,O-oversulfated K5 with a degree of sulfation of from 3.2 to 4.

104. Use according to claim 3 wherein said N,O-oversulfated K5 has a degree of sulfation from 3.5 to 4.

5. Use according to claim 3 wherein said N,O-oversulfated K5 has a degree of sulfation of from 3.7 to 4.

6. Use according to anyone of claims 1-5 wherein said angiogenesis–dependent pathologies include diabetic retinopathy, neovascularization of the implanted cornea, neovascular glaucoma, trachoma, retrolental fibrodisplasia, psoriasis, pyogenic granuloma, development of the aterosclerotic plaque, hemangioma and angiofibroma, atero-venous malformations, arthritis, and solid tumours.

207. Use according to anyone of claims 1-6 wherein the pharmaceutically acceptable salt is selected from the group consisting of sodium, potassium, calcium, magnesium aluminium and zinc salts.

8. Use according to anyone of claims 1-7 wherein said N,O sulfated K5 has a molecular weight with a distribution from about 2,000 to about 16,000.

259. Use according to claim 8 wherein said distribution is from about 2,500 to about 10,000, with a mean molecular weight of about 6,500.

10. Use according to anyone of claims 1-7 wherein said N,O sulfated K5 has a molecular weight with a distribution of from about 13,000 to about 65,000.

11. Use according to claim 10 wherein said distribution is from about 25,000 to about 50,000 with a mean molecular weight of about 40,000.

12. Use according to anyone of claims 1-7 wherein said N,O-sulfated K5 has a molecular weight with a distribution from about 2,000 to about 65,000, with a mean molecular weight of 25,000-30,000.

13. Use according to anyone of claims 1-7 wherein said N,O-sulfated K5 is obtained by depolymerization and has a mean molecular weight of from 2,000 to 5,000.

14. Pharmaceutical composition for the treatment of angiogenesis-dependent pathologies which comprises, as an active ingredient thereof, a pharmacologically effective amount of a N,O-sulfated K5 having a degree of sulfation of at least 2, or of one of its pharmaceutically acceptable salts, in admixture with a carrier or pharmaceutical excipient.

15. Pharmaceutical composition according to claim 14 which is in dosage units.

16. Pharmaceutical composition according to anyone of claims 14 and 15 wherein said N,O-sulfated K5 has a degree of sulfation of from 2.5 to 4.

17. Pharmaceutical composition according to anyone of claims 14 and 15 wherein said N,O-sulfated K5 is a N,O-oversulfated K5 with degree of sulfation of from 3.2 to 4.

18. Pharmaceutical composition according to claim 17 wherein said N,O-oversulfated K5 has a degree of sulfation of from 3.5 to 4.

19. Pharmaceutical composition according to claim 17 wherein said N,O-oversulfated K5 has a degree of sulfation of from 3.7 to 4.

2520. Pharmaceutical composition according to anyone of claims 14-19 wherein said N,O-sulfated K5 has a molecular weight with a distribution from about 2,000 to about 16,000.

21. Pharmaceutical composition according to claim 20 wherein said distribution is from about 2,500 to about 10,000, with a mean molecular

weight of about 6,500.

22. Pharmaceutical composition according to anyone of claims 14-19 wherein said N,O-sulfated K5 has a molecular weight with a distribution from about 13,000 to about 65,000.

523. Pharmaceutical composition according to claim 22 wherein said distribution is from about 25,000 to about 50,000 with a mean molecular weight of about 40,000.

24. Pharmaceutical composition according to anyone of claims 14-19 wherein said N,O-sulfated K5 has a molecular weight with a distribution from about 2,000 to about 65,000 with a mean molecular weight of 25,000-30,000.

25. Pharmaceutical composition according to claim 24 wherein said N,O-sulfated K5 is obtained by depolymerization and has a mean molecular weight of from 2,000 to 5,000.

26. Method for the treatment of the angiogenesis-dependent pathologies which comprises administering an effective amount of a N,O-sulfated K5 with a degree of sulfation of at least 2 to the patient in need of said treatment.

27. Method according to claim 26 wherein said treatment is performed administering a pharmaceutical composition according to claims 14-25 to said patient.

2028. Method according to claim 27 wherein said pharmaceutical composition is formulated with carriers or pharmaceutically acceptable diluents for parenteral administration or topic application.

29. Method according to claim 28 wherein a dosage of from 0.5 to 500 mg/Kg/day is provided for the parenteral administration and a dosage of from 1 to 1,000 mg /Kg/day is provided for the topic application.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/[redacted]2/01138

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/737 A61P29/00 A61P35/00 A61P17/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 09636 A (TORRI GIANGIACOMO ;NAGGI ANNAMARIA (IT); ISTITUTO SCIENT DI CHIMIC) 12 March 1998 (1998-03-12) cited in the application	1,2, 6-16, 20-29
Y	See antimetastatic activity (ex. 8). claims 1-4; examples ---	1-29
X	WO 92 17507 A (ITALFARMACO SPA ;MAX PLANCK INST FUER IMMUNBIOL (DE); TUBBY DAVID) 15 October 1992 (1992-10-15) cited in the application examples 8-12 --- -/--	14-16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 July 2002	22/07/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Veronese, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	LEALI D ET AL: "FIBROBLAST GROWTH FACTOR-2 ANTAGONIST ACTIVITY AND ANGIOSTATIC CAPACITY OF SULFATED ESCHERICHIA COLI K5 POLYSACCHARIDE DERIVATIVES" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, US, vol. 41, no. 276, 12 October 2001 (2001-10-12), pages 37900-37908, XP001077702 ISSN: 0021-9258 See dresults and discussion abstract; figures; tables	1-29
X	CASU B ET AL: "Heparin-like compounds prepared by chemical modification of capsular polysaccharide from E. coli K5" CARBOHYDRATE RESEARCH, ELSEVIER SCIENTIFIC PUBLISHING COMPANY. AMSTERDAM, NL, vol. 263, no. 2, 17 October 1994 (1994-10-17), pages 271-284, XP004022216 ISSN: 0008-6215 cited in the application See derivative C1-C4. table 1	14-16
Y	See page 279, "procedure C" and sequence N.8	1-29
Y	FOLKMAN J ET AL: "CONTROL OF ANGIOGENESIS BY HEPARIN AND OTHER SULFATED POLYSACCHARIDES" ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY, SPRING ST., NY, US, vol. 313, 1992, pages 355-364, XP000952102 ISSN: 0065-2598 page 353; figures See page 360 "Summary"	1-29
Y	HAHNENBERGER R ET AL: "Low-sulphated oligosaccharides derived from heparan sulphate inhibit normal angiogenesis." GLYCOBIOLOGY. ENGLAND DEC 1993, vol. 3, no. 6, December 1993 (1993-12), pages 567-573, XP002204072 ISSN: 0959-6658 the whole document	1-29

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INTERNATIONAL SEARCH REPORT

Internat[redacted]al Application No
PCT/I[redacted]2/01138

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 98 34958 A (CIPOLLETTI GIOVANNI ;PASQUA ORESTE (IT); INALCO SPA (IT); ZOPPETTI) 13 August 1998 (1998-08-13) cited in the application page 2, line 10-12 page 4, line 1-3 ---	1-29
Y	KASBAUER C W ET AL: "Sulfated beta-(1->4)-galacto-oligosaccharides and their effect on angiogenesis" CARBOHYDRATE RESEARCH, ELSEVIER SCIENTIFIC PUBLISHING COMPANY. AMSTERDAM, NL, vol. 330, no. 3, 15 February 2001 (2001-02-15), pages 427-430, XP004230253 ISSN: 0008-6215 the whole document -----	1-29

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB 02/01138

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 26-29 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/I[redacted]/01138

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9809636	A	12-03-1998	IT	MI961840 A1	06-03-1998
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			EP	0958307 A1	24-11-1999
			JP	2001510502 T	31-07-2001
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